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(54) ALFA-L-RHAMNOSIDASE AND ITS PRODUCTION
(57)Abstract:
PROBLEM TO BE SOLVED: To obtain a new α-L-rhamnosidase originated from a microorganism of the genus Pichia, having activity to release p-nitrophenol by using p-nitrophenyl-α-Lrhamnopyranoside as a substrate, having glucose tolerance, useful for wine fermentation or the like.
SOLUTION: This new α-L-rhamnosidase has the following features: p- nitrophenol is released by acting it on p-nitrophenyl-α-L-rhamnopyranoside as a substrate; when the substrate is made to react for 10 min at 30°C, the optimum pH is 6.0-7.0; when held for 30 min at 30°C, thecc-L-rhamnosidase exhibits stable enzymatic activity at pH 5.0-7.0; when the substrate is made to react for 10 min at pH 6.5, the optimum temperature is about 40°C; when held for 30 min at pH 6.5, the α-L-rhamnosidase is stable up to 20°C and gradually deactivated with the exceeding of the temperature; the molecular weight is about 88,000 daltons (SDS-PAGE); and the α-L-rhamnosidase has glucose tolerance and ethanol tolerance. This enzyme increases fragrance components in wine fermentation, and it is useful for improving the quality of wine or the like. The enzyme is obtained from the fermentation product of a microorganism of the genus Pichia.

CLAIMS

[Claim(s)]
[Claim 1]Alpha-L-rhamnosidase which has the following character.
(1) OPERATION -- when :p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.
(2) Optimal pH : optimal pH at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 ** is in the 6.0 to 7.0 neighborhood.
(3) pH stability : when it holds for 30 minutes at 30 **, enzyme activity stable in pH 5.0-7.0 is shown.
(4) Optimum temperature : optimum temperature at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 ** pH 6.5 is about 40 **.
(5) Thermal stability : when it holds for 30 minutes in pH6.5, if enzyme activity stable to 20 ** is shown and it is exceeded, it will be deactivated gradually.
(6) Inhibitor : mercury para-chlorobenzoate, mercuric chloride, and a cupric chloride receive inhibition, and it is hardly prevented in ethylenediaminetetraacetic acid and mercaptoethanol.

(7) A molecular weight : a molecular weight measured by an SDS-polyacrylamide-gel-electrophoresis method is about 88,000 dalton.
(8) An isoelectric point: An isoelectric point by isoelectric focusing is pI4.9.
(9) Glucose tolerance : 76% of relative activity is shown in 500mM glucose.
(10) Ethanol resistance : 67% of relative activity is shown in 20 capacity % ethanol.
[Claim 2]A manufacturing method of alpha-L-rhamnosidase extracting the alpha-L-rhamnosidase according to claim 1 from a culture produced by cultivating a microorganism belonging to the Pichia (Pichia) group.
[Claim 3]A manufacturing method of alpha-L-rhamnosidase extracting the alpha-L-rhamnosidase according to claim 1 from a culture produced by cultivating a microorganism belonging to the Pichia (Pichia) group by a culture medium containing rhamnose (rhamnose).
[Claim 4]The Pichia ANGUSUTA (Pichia angusta)X349 share which has the alpha-L-rhamnosidase production ability according to claim 1 (FERM P-17211).

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention relates to the microorganism which produces new alpha-L-rhamnosidase, its manufacturing method, and its enzyme.
[0002]
[Description of the Prior Art]Linalool (linalool), geraniol (geraniol) which are contained in a grape, Monoterpenes, such as Nellore (nerol), citronellol (citronelol), and the alpha-terpineol (terpineol), have contributed to the scent of a grape or wine greatly. Although such monoterpenes are contained also as fragrant educt, the many exist as a glycoside which does not have fragrance.
[0003]Many of these glycosides 6-O-alpha-L-arabinofuranosyl beta-D-glucopyranoside (6-O-alpha-L-arabinofuranosyl-beta-D-glucopyranosides), 6-O-alpha-L-rhamnopyranosyl beta-D-glucopyranoside (6-O-alpha-L-rhamnopyranosyl-beta-D-glucopyranosides), It is known that they are jig RIKOSHIDO called 6-O-beta-D-apiofuranosyl beta-D-glucopyranoside (6-O-beta-D-apiofuranosyl-beta-D-glucopyranosides).
[0004]If the hydrolysis reaction of these jig RIKOSHIDO is carried out enzymatically, fragrant monoterpenes will separate. This hydrolysis reaction is performed in two steps. First Namely, L-rhamnosidase (alpha-L-rhamnosidase), Alpha-L-arabino flannel SHIDAZE (alpha-L-arabinofuranosidase), A glycosidic linkage is cut by beta-APIOSHIDAZE (beta-apiosidase) etc. and mono- **********- beta-D-glucoside (monoterpenyl-beta-D-glucosides) corresponding, respectively is produced. Subsequently, beta-D-glucosidase (beta-D-glucosidase) acts on this mono- **********- beta-D-glucoside. Alpha-L-rhamnosidase is an enzyme which hydrolyzes the alpha-L-rhamnopyranoside residue of the nonreducing terminal of the polysaccharide containing alpha-L-rhamnose (L-rhamnose) among the hydrolase which carries out the catalyst of the first-step reaction. Therefore, by using alpha-L-rhamnosidase with beta-D-glucosidase etc., a perfume component can be made to be able to increase in wine brewing, and the quality of wine can be raised.
[0005]However, in using these enzymes for wine brewing, there is a problem which many should conquer. It is that it is required for one to have enzyme activity also under

existence of glucose or ethanol. In the case of the enzyme of bacteria origin, by a biomass external secretion [of that there is a good rare colander bacteria smell and mold origin] type crude enzyme, there is a problem that a good rare colander side reaction occurs for other catalytic activity in one. Although there is a report about these bacteria or alpha-L-rhamnosidase of mold origin, on the other hand, there is no report about the thing of yeast origin. If it says strictly, the thing of Saccharomyces cerevisiae (Saccharomyces cerevisiae) origin is reported, but the main catalytic activity is beta-D-glucosidase and is only also having alpha-L-rhamnosidase activity slightly.

[0006]

[Problem(s) to be Solved by the Invention]Therefore, the purpose of this invention is to provide the microorganism which produces new alpha-L-rhamnosidase provided with the characteristic which can be used for wine brewing, its manufacturing method, and its enzyme.

[0007]

[Means for Solving the Problem]This invention persons ask for a microorganism which can produce alpha-L-rhamnosidase provided with the characteristic which can be used for wine brewing, As a result of searching centering on yeast with until now few examples of a report especially, one strain belonging to the Pichia (Pichia) group finds out producing new alpha-L-rhamnosidase, and came to complete this invention. Alpha-L-rhamnosidase activity is measured by a measuring method which crushes a biomass and for which a centrifugal supernatant liquid fraction is later mentioned after culture by a culture medium which specifically makes L-rhamnose the only carbon source for many microorganisms isolated from various kinds of sources of separation, and the target microorganism is chosen.

[0008]That is, according to this invention, alpha-L-rhamnosidase having the following character is provided.

(1) OPERATION -- when :p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.

(2) Optimal pH : optimal pH at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 ** is in the 5.0 to 7.0 neighborhood.

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(4) Optimum temperature : optimum temperature at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 ** pH 6.5 is about 40 **.

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[0009](6) Inhibitor : mercury para-chlorobenzoate, mercuric chloride, and a cupric chloride receive inhibition, and it is hardly prevented in ethylenediaminetetraacetic acid and mercaptoethanol.

(7) Molecular weight : a molecular weight measured by an SDS-polyacrylamide-gel-electrophoresis method is about 88,000 dalton.

(8) Isoelectric point: An isoelectric point by isoelectric focusing is pI4.9.

(9) Glucose tolerance : 76% of relative activity is shown in 500mM glucose.

(10) Ethanol resistance : 67% of relative activity is shown in 20 capacity % ethanol.

[0010]A manufacturing method of alpha-L-rhamnosidase of this invention is the method of acquiring alpha-L-rhamnosidase from culture medium obtained by belonging to the

Pichia (Phicia) group and cultivating a microorganism which produces alpha-L-rhamnosidase.

[0011]Character of a separation strain to produce alpha-L-rhamnosidase of this invention is shown below.

A. Form a colony of morphometrical characteristic 1 opalescence.

2) Germination performs asexual growth.

3) Form 1 thru/or 4 ascospores of a bowler hat form by a glucose malt culture medium.

[0012]

B. physiological character 1 fermentability Glucose + galactose . - malt sugar - sucrose - trehalose . + melibiose - lactose -2 utilization nature . glucose + lactose - galactose . - raffinose - sorbose - ribitol . + ribose + mannitol + xylose + inositol - rhamnose + methanol + sucrose + ethanol + malt sugar + nitrate + melibiose - (+:utilization carries out -:utilization does not carry out)

[0013]It refers to BARNETT & PAYNE YARROW YEASTS:Characteristicsand identification (Second edition) based on the above mycology character, This strain was identified to Pichia ANGUSUTA (Pichia angusta). It was judged that it was a new strain from a point of producing above-mentioned new alpha-L-rhamnosidase. Therefore, it was named Pichia ANGUSUTA (Pichia angusta) X349, and a bacteria stock was deposited with National Institute of Bioscience and Human-Technology. A deposition number is FERM P-17211. If a microorganism used for this invention is a strain which produces alpha-L-rhamnosidase of this invention by a microorganism belonging to a Pichia group besides said Pichia ANGUSUTA (Pichia angusta) X349, it will not interfere.

[0014]In this invention, it may be used for culture of the usual microorganism as a culture medium which produces alpha-L-rhamnosidase, and especially if a microorganism used grows, it will not be restricted. As a carbon source, although sucrose etc. can be used, for example, a point of producing a lot of alpha-L-rhamnosidase to especially L-rhamnose is desirable. As a nitrogen source, peptone is raised, for example. To a culture medium, mineral salt, for example, sodium chloride, besides a carbon source and a nitrogen source can be added. Although the culture temperature should just be a temperature which a microorganism to be used grows, near [good] 28 ** of growth is preferred. Initiation pH of a culture medium to be used has the 4.0 preferred neighborhoods. Alpha-L-rhamnosidase is accumulated into culture medium at culture time by [for growth of the bacillus concerned, and alpha-L-rhamnosidase production / sufficient] usually cultivating for 48 hours, although time continuation is carried out.

[0015]Biomasses are collected with filtration, centrifugal separation, or filtration after culture, and supernatant liquid produced by crushing a biomass by mechanical disruption processing of lytic enzyme processing or ultrasonication, an French press, a homogenizer, etc., and centrifuging is used as crude enzyme liquid. Although crude enzyme liquid can also be used as it is, separation refinement can be carried out according to the separation refinement methods of common enzymes, such as ammonium sulfate curing salting and a solvent sedimentation method. An enzyme preparation with high activity purity can also be prepared combining suitably a refining means of the usual enzymes, such as ion exchange chromatography and gel filtration chromatography. The character of this invention enzyme obtained by refining combining various chromatography methods from crude enzyme liquid is as follows. Measurement of enzyme activity was performed to below by a following method.

[0016][Enzyme activity measurement] 0.2 ml of 2mM p-nitrophenyl alpha-L-rhamnopyranoside solutions which dissolved in the buffer solution are mixed to 0.2 ml of enzyme sample solutions diluted with 50mM sodium phosphate buffer solution (pH 6.5), and it is made to react to them for 10 minutes at 30 **. Then, 1.6 ml of 1M sodium carbonate is added, a reaction is stopped, and p-nitrophenol separated by measuring an absorbance at 405 nm is quantified. One enzyme unit (U) which shows enzyme activity was defined in the above-mentioned conditions, i.e., 30 **, and pH 6.5 as an amount of enzymes which makes p-nitrophenol of 1micromol generate in 1 minute after p-nitrophenyl alpha-L-rhamnopyranoside.

[0017](1) OPERATION -- when :p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.

Optimal pH : (2) p-nitrophenyl alpha-L-rhamnopyranoside is used as a substrate, In accordance with said enzyme activity measurement, relative activity was measured except using a 50mM citrate-phosphate buffer solution (pH 3.0-7.0), 50mM phosphate buffer solution (pH 8.0-9.0), and 50mM glycine sodium hydroxide buffer solution (pH 10.0) as buffer solution. The result is as being shown in drawing 1, and reaction optimal pH is in the 6.0 to 7.0 neighborhood.

pH stability : (3) A 50mM citrate-phosphate buffer solution (pH 3.0-7.0), After holding for 30 minutes at 30 ** in each buffer solution of 50mM phosphate buffer solution (pH 8.0-9.0) and 50mM glycine sodium hydroxide buffer solution (pH 10.0), residual activity was measured in accordance with said enzyme activity measurement. The result is as being shown in drawing 1, and a stable pH range is pH 5.0-7.0.

(4) Optimum temperature : in accordance with said enzyme activity measurement, relative activity was measured at various temperature. The result is as being shown in drawing 2, and optimum temperature is about 40 **.

(5) Thermal stability : after holding for 30 minutes in 50mM phosphate buffer solution (pH 6.5), residual activity was measured in accordance with said enzyme activity measurement. As a result, if this enzyme shows enzyme activity stable to 20 ** and it is exceeded as shown in drawing 2, it will be deactivated gradually.

[0018](6) Influence of various reagents : influence on this enzyme of various reagents was investigated. That is, various reagents shown in Table 1 were dissolved so that it might become 50mM phosphate buffer solution (pH 6.5) with 1mM, an enzyme solution was mixed to this, residual enzyme activity after holding for 10 minutes in 30 ** was measured in accordance with said enzyme activity measurement, and a result of Table 1 was obtained.

[Table 1]

[0019](7) Molecular weight : it is SDS-polyacrylamide gel electrophoresis () about this refined enzyme. [PhastSystem and] A molecular weight which measured by performing PhastGel homogeneous12.5 and PhastGel SDS bufferstrips (Pharmacia manufacture) is about 88,000 dalton. To a standard of a molecular weight, b type phosphorylase (molecular weight of 94,000 dalton), Bovine serum albumin (molecular weight of 67,000 dalton), ovalbumin (molecular weight of 43,000 dalton), Carbonic anhydro RAZE (molecular weight of 30,000 dalton), soybean typsin inhibitor (molecular weight of 20,100 dalton), and lactalbumin (molecular weight of 14,400 dalton) were used.

(8) Isoelectric point: The isoelectric point which measured about this refined enzyme by performing isoelectric focusing (PhastSystem, PhastGel IEF 3-9 (Pharmacia manufacture)) is pI4.9. To the standard of an isoelectric point, horse myoglobin (acidic:pI6.85), Homo sapiens carbonic anhydro RAZEB (pI6.55), cow carbonic anhydro

RAZEB (pI5.85), the beta-lactoglobulin A (pI5.20), a soybean trypsin inhibitor (pI4.55), and amyloglucosidase (pI3.50) were used.

[0020](9) Glucose tolerance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing glucose of 0 - 500mM concentration. The result is as being shown in drawing 3, and shows 76% of relative activity also in 500mM glucose.

(10) Ethanol resistance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing the ethanol of 0 - 20 capacity % concentration. It is as being shown in drawing 4, and in 10 capacity % ethanol, the result shows 85% of relative activity, and shows 67% of relative activity in 20 capacity % ethanol.

[0021]

[Example]Next, although the example of this invention is given and this invention is explained in detail, the example shown below is an example and does not limit the technical scope of this invention.

Example 1 : Culture of a strain, and 1.0% of production L-rhamnose of alpha-L-rhamnosidase, Peptone 0.5%, the culture medium which consists of 0.3% of a yeast extract and 0.3% of a malt extract was adjusted the pH to 4.0 with chloride, 100 ml of this was taught to each 40 Erlenmeyer flasks of 500-ml **, and autoclaving was carried out for 15 minutes 120 **. The Pichia ANGUSUTA (Pichia angusta)X349 share was inoculated into this, and it cultivated on the rotation agitator for two days at 28 ** and 220 rpm. The obtained culture medium 4L is centrifuged (for 1000xg and 10 minutes), biomasses are collected, and it is 20mM. A screw/tris buffers (pH 6.5) washed twice. This was suspended to the buffer solution, the 2.5-times the amount glass bead (0.45-0.55 mm in diameter) of biomass weight was added, and the biomass was crushed. Except for biomass debris, the cell extract was obtained by centrifugal separation (for 1000xg and 10 minutes). Ultracentrifuge processing (for 100,000xg and 90 minutes) of this was carried out, and it separated into the microsome fraction and the supernatant liquid fraction. Since all the activity existed in the supernatant liquid fraction when such fraction alpha-L-rhamnosidase activity was measured, the refining operation described below was presented with the supernatant liquid fraction.

[0022]Example 2: Especially refining operation shown below in refining of an enzyme was altogether performed at 4 **, unless it refused.

(1) Ammonium sulfate precipitate refined 600 ml of supernatant liquid fractions obtained in ammonium sulfate precipitate example 1. the precipitate formed from saturation in the fraction of saturation 80% 30% -- centrifugal separation (1,000xg.) It carried out for 30 minutes, collected, dissolved in the 100mM sodium acetate buffer solution (pH 6.0) containing 1M NaCl, 1mM MnCl2, 1mM CaCl2, and 0.003mM NaN3, and dialyzed to the buffer solution.

(2) The enzyme solution which carried out concanavalin A-sepharose column chromatography dialysis was given to the concanavalin A-sepharose column (phi2.6x6.5cm) beforehand equilibrated with the buffer solution. Since the target enzyme did not stick to this column, it was eluted with the buffer solution. Activity fractions are collected, and it condenses with ultrafiltration equipment (made by PM-10:Amicon), and is 20mM. It dialyzed to a screw/tris buffers (pH 6.5).

(3) DEAE Bio-Gel A They are 20mM beforehand about the enzyme solution which carried out agarose column chromatography dialysis. The DEAE Bio-Gel A agarose column (phi2.6x6.0cm) equilibrated with a screw/tris buffers (pH 6.5) was given. After

the buffer solution washed, it was eluted by the sodium chloride linear gradient (0-0.3M) using the buffer solution. Activity fractions were collected and it dialyzed to 50mM sodium acetate buffer solution (pH 5.0).

[0023](4) An arabinose sepharose 6B affinity column chromatography rhamnose sepharose 6B affinity carrier, In accordance with how to recommend a manufacturing company for the epoxy activation sepharose 6B (made by Pharmacia biotech AB), rhamnose was combined and it prepared. The enzyme liquid obtained by the DEAE Bio-GelA agarose column chromatography mentioned above, The rhamnose sepharose 6B affinity column (phi1.5x7.2cm) beforehand equilibrated with 50mM sodium acetate buffer solution (pH 5.0) was given, and the buffer solution washed. Next, it was eluted with the buffer solution containing 1M NaCl, activity fractions were collected, and it dialyzed to distilled water.

(5) The enzyme solution which carried out hydroxyapatite column chromatography dialysis, The hydroxyapatite column (phi1.5x2.8cm) beforehand equilibrated with 10mM sodium phosphate buffer solution was given, the buffer solution washed enough, the back was eluted by the sodium phosphate linear gradient (10 - 200mM), and activity fractions were collected.

[0024]The activity of the enzyme in every above purification process is shown in Table 2. Cow serum albumin is used for protein as a standard substance, and it is the bottom in fixed quantity by the BURADOFODO method (Bradfordmethod).

[Table 2]

rha-sepharose 6B: Rhamnose sepharose 6B[0025]Example 3: The decomposition examination of the various chromophoric substrates shown in Table 3 was done using the refining enzymes obtained in substrate specificity example 2. The decomposition examination was done according to the method of said enzyme activity measurement, and the existence of activity was investigated by generation of p-nitrophenol. A result is shown in Table 3.

[Table 3]

[0026]Although this enzyme acts on p-nitrophenyl alpha-L-rhamnopyranoside and p-nitrophenol is separated, It hardly acts on p-nitrophenyl alpha-L-arabino furanoside, p-nitrophenyl beta-D-glucopyranoside, p-nitrophenyl beta-D-xylopyranoside, and p-nitrophenyl beta-D-cello BIOSHIDO, but it turns out that it is an enzyme with high substrate specificity.

[0027]1000 ml of grape juice (muscat OBU Alexandria kind) adjusted the pH to 7.0 by operation sodium hydroxide to the glycosides of Example 4:grape juice origin -- a synthetic adsorption resin column (phi -- 22 mm x 350 mm) Amberlite XAD-2: It let it pass to made in loam and Haas. 500 ml of distilled water washed this, and water soluble compounds were removed. After removing through and free terpenes for 1000 ml of pentane-ether (1:1) furthermore, glycosides were made eluted with 1000 ml of methanol. Concentration hardening by drying of this fraction was carried out under 30 ** and decompression, and 100 mg of glycosides were obtained. 10 mg of the above-mentioned glycosides were dissolved in 2 ml (0.5U, 50mM sodium phosphate buffer solution (pH 6.5)) of refining enzyme solutions obtained in Example 2, and it was made to react for two days at 30 **. After 5 ml of pentane-ether (1:1) having extracted the separated terpenes twice and condensing it, it analyzed on condition of the following using capillary gas chromatography. A result is shown in Table 4. Thus, the enzyme of this invention disassembles the glycosides of grape juice, and has the outstanding character to which a perfume component is made to increase notably.

[Table 4]

[0028](Analysis condition)

device: -- Hewlett Packard 5980 column: -- a DB-WAX capillary column (made by phi0.25-mmx30 m:J&WScientific)

detector: -- FID helium flow **: -- a part for 2.0-ml/-- temperature program: -- -> with a temperature up of 75 ** 220 ** (a part for 4 **/), and 220 ** (15 minutes) of maintenance sample injection section temperature: -- 230 ** primary detecting element temperature: -- 220 ** internal standard: -- amount of 2-ethyl-1-hexanol samples: -- 2microL

[0029]Example 5 :. Have a rhamnosyl group in the operation nonreducing terminal to the rhamnosyl glycoside of natural flavonoid. The decomposition product of naringin (narigin), rutin (rutin), hesperidin (hesperidin), and the rhamnosyl glycoside of quercitrin (quercitrin) was analyzed using silica gel thin layer chromatography. That is, the reaction solution in which 0.5 ml of solutions (0.5U, 50mM sodium phosphate buffer solution pH6.5) of the refining enzymes obtained in Example 2 were made to dissolve 5 mg of substrates was held at 30 ** overnight. This sample was given to 4.0microl and a silica gel thin layer (DC-Alufolien Kieselgel 60: made by Merck Co.). After developing using acetonitrile distilled water (5:1, V/V) mixture, using rhamnose and glucose as a standard substance, the ethanol solution of sulfuric acid was sprayed 10%, it heated for 5 minutes at 105 **, and sugars were detected.

[0030]As a result, naringin (narigin), the rutin (rutin), and the rhamnosyl glycoside of hesperidin (hesperidin) received decomposition by alpha-L-rhamnosidase, and rhamnose was detected. On the other hand, rhamnose was not detected about the rhamnosyl glycoside of quercitrin (quercitrin).

[0031]

[Effect of the Invention]this invention enzyme has the activity which was dramatically excellent under high-concentration glucose and a high-concentration ethanol existence. It can use for brewing of wine.

That is, by using this invention enzyme with beta-D-glucosidase etc., a perfume component can be made to be able to increase in wine brewing, and the quality of wine can be raised. On the other hand, it does not have an adverse effect on the flavor of wine by work of a bacteria smell or a contamination enzyme. It can use not only for grape juice but for the fruit juice of citruses from the size of substrate specificity to the rhamnosyl glycoside of natural flavonoid of this enzyme.

TECHNICAL FIELD

[Field of the Invention]This invention relates to the microorganism which produces new alpha-L-rhamnosidase, its manufacturing method, and its enzyme.

PRIOR ART

[Description of the Prior Art]Linalool (linalool), geraniol (geraniol) which are contained in a grape, Monoterpenes, such as Nellore (nerol), citronellol (citronelol), and the alpha-terpineol (terpineol), have contributed to the scent of a grape or wine greatly. Although such monoterpenes are contained also as fragrant educt, the many exist as a glycoside which does not have fragrance.

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[0005]However, in using these enzymes for wine brewing, there is a problem which many should conquer. It is that it is required for one to have enzyme activity also under existence of glucose or ethanol. In the case of the enzyme of bacteria origin, by a biomass external secretion [of that there is a good rare colander bacteria smell and mold origin] type crude enzyme, there is a problem that a good rare colander side reaction occurs for other catalytic activity in one. Although there is a report about these bacteria or alpha-L-rhamnosidase of mold origin, on the other hand, there is no report about the thing of yeast origin. If it says strictly, the thing of Saccharomyces cerevisiae (Saccharomyces cerevisiae) origin is reported, but the main catalytic activity is beta-D-glucosidase and is only also having alpha-L-rhamnosidase activity slightly.

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(4) Optimum temperature : optimum temperature at a time of using p-nitrophenyl alpha-L-rhamnopyranoside as a substrate and making it react for 10 minutes at 30 ** pH 6.5 is about 40 **.

(5) Thermal stability : when it holds for 30 minutes in pH6.5, if enzyme activity stable to 20 ** is shown and it is exceeded, it will be deactivated gradually.

[0009](6) Inhibitor : mercury para-chlorobenzoate, mercuric chloride, and a cupric chloride receive inhibition, and it is hardly prevented in ethylenediaminetetraacetic acid and mercaptoethanol.

(7) Molecular weight : a molecular weight measured by an SDS-polyacrylamide-gel-electrophoresis method is about 88,000 dalton.

(8) Isoelectric point: An isoelectric point by isoelectric focusing is pI4.9.

(9) Glucose tolerance : 76% of relative activity is shown in 500mM glucose.

(10) Ethanol resistance : 67% of relative activity is shown in 20 capacity % ethanol.

[0010]A manufacturing method of alpha-L-rhamnosidase of this invention is the method of acquiring alpha-L-rhamnosidase from culture medium obtained by belonging to the Pichia (Phicia) group and cultivating a microorganism which produces alpha-L-rhamnosidase.

[0011]Character of a separation strain to produce alpha-L-rhamnosidase of this invention is shown below.

A. Form a colony of morphometrical characteristic 1 opalescence.

2) Germination performs asexual growth.

3) Form 1 thru/or 4 ascospores of a bowler hat form by a glucose malt culture medium.

[0012]

B. physiological character 1 fermentability Glucose + galactose . - malt sugar - sucrose - trehalose . + melibiose - lactose -2 utilization nature . glucose + lactose - galactose . - raffinose - sorbose - ribitol . + ribose + mannitol + xylose + inositol - rhamnose +

methanol + sucrose + ethanol + malt sugar + nitrate + melibiose - (+:utilization carries out -:utilization does not carry out)

[0013]It refers to BARNETT & PAYNE YARROW YEASTS:Characteristicsand identification (Second edition) based on the above mycology character, This strain was identified to Pichia ANGUSUTA (Pichia angusta). It was judged that it was a new strain from a point of producing above-mentioned new alpha-L-rhamnosidase. Therefore, it was named Pichia ANGUSUTA (Pichia angusta) X349, and a bacteria stock was deposited with National Institute of Bioscience and Human-Technology. A deposition number is FERM P-17211. If a microorganism used for this invention is a strain which produces alpha-L-rhamnosidase of this invention by a microorganism belonging to a Pichia group besides said Pichia ANGUSUTA (Pichia angusta) X349, it will not interfere.

[0014]In this invention, it may be used for culture of the usual microorganism as a culture medium which produces alpha-L-rhamnosidase, and especially if a microorganism used grows, it will not be restricted. As a carbon source, although sucrose etc. can be used, for example, a point of producing a lot of alpha-L-rhamnosidase to especially L-rhamnose is desirable. As a nitrogen source, peptone is raised, for example. To a culture medium, mineral salt, for example, sodium chloride, besides a carbon source and a nitrogen source can be added. Although the culture temperature should just be a temperature which a microorganism to be used grows, near [good] 28 ** of growth is preferred. Initiation pH of a culture medium to be used has the 4.0 preferred neighborhoods. Alpha-L-rhamnosidase is accumulated into culture medium at culture time by [for growth of the bacillus concerned, and alpha-L-rhamnosidase production / sufficient] usually cultivating for 48 hours, although time continuation is carried out.

[0015]Biomasses are collected with filtration, centrifugal separation, or filtration after culture, and supernatant liquid produced by crushing a biomass by mechanical disruption processing of lytic enzyme processing or ultrasonication, an French press, a homogenizer, etc., and centrifuging is used as crude enzyme liquid. Although crude enzyme liquid can also be used as it is, separation refinement can be carried out according to the separation refinement methods of common enzymes, such as ammonium sulfate curing salting and a solvent sedimentation method. An enzyme preparation with high activity purity can also be prepared combining suitably a refining means of the usual enzymes, such as ion exchange chromatography and gel filtration chromatography. The character of this invention enzyme obtained by refining combining various chromatography methods from crude enzyme liquid is as follows. Measurement of enzyme activity was performed to below by a following method.

[0016][Enzyme activity measurement] 0.2 ml of 2mM p-nitrophenyl alpha-L-rhamnopyranoside solutions which dissolved in the buffer solution are mixed to 0.2 ml of enzyme sample solutions diluted with 50mM sodium phosphate buffer solution (pH 6.5), and it is made to react to them for 10 minutes at 30 **. Then, 1.6 ml of 1M sodium carbonate is added, a reaction is stopped, and p-nitrophenol separated by measuring an absorbance at 405 nm is quantified. One enzyme unit (U) which shows enzyme activity was defined in the above-mentioned conditions, i.e., 30 **, and pH 6.5 as an amount of enzymes which makes p-nitrophenol of 1micromol generate in 1 minute after p-nitrophenyl alpha-L-rhamnopyranoside.

[0017](1) OPERATION -- when :p-nitrophenyl alpha-L-rhamnopyranoside is made to act as a substrate, separate p-nitrophenol.

Optimal pH : (2) p-nitrophenyl alpha-L-rhamnopyranoside is used as a substrate, In accordance with said enzyme activity measurement, relative activity was measured except using a 50mM citrate-phosphate buffer solution (pH 3.0-7.0), 50mM phosphate buffer solution (pH 8.0-9.0), and 50mM glycine sodium hydroxide buffer solution (pH 10.0) as buffer solution. The result is as being shown in drawing 1, and reaction optimal pH is in the 6.0 to 7.0 neighborhood.

pH stability : (3) A 50mM citrate-phosphate buffer solution (pH 3.0-7.0), After holding for 30 minutes at 30 ** in each buffer solution of 50mM phosphate buffer solution (pH 8.0-9.0) and 50mM glycine sodium hydroxide buffer solution (pH 10.0), residual activity was measured in accordance with said enzyme activity measurement. The result is as being shown in drawing 1, and a stable pH range is pH 5.0-7.0.

(4) Optimum temperature : in accordance with said enzyme activity measurement, relative activity was measured at various temperature. The result is as being shown in drawing 2, and optimum temperature is about 40 **.

(5) Thermal stability : after holding for 30 minutes in 50mM phosphate buffer solution (pH 6.5), residual activity was measured in accordance with said enzyme activity measurement. As a result, if this enzyme shows enzyme activity stable to 20 ** and it is exceeded as shown in drawing 2, it will be deactivated gradually.

[0018](6) Influence of various reagents : influence on this enzyme of various reagents was investigated. That is, various reagents shown in Table 1 were dissolved so that it might become 50mM phosphate buffer solution (pH 6.5) with 1mM, an enzyme solution was mixed to this, residual enzyme activity after holding for 10 minutes in 30 ** was measured in accordance with said enzyme activity measurement, and a result of Table 1 was obtained.

[Table 1]

[0019](7) Molecular weight : it is SDS-polyacrylamide gel electrophoresis () about this refined enzyme. [PhastSystem and] The molecular weight which measured by performing PhastGel homogeneous12.5 and PhastGel SDS bufferstrips (Pharmacia manufacture) is about 88,000 dalton. To the standard of a molecular weight, b type phosphorylase (molecular weight of 94,000 dalton), Bovine serum albumin (molecular weight of 67,000 dalton), ovalbumin (molecular weight of 43,000 dalton), Carbonic anhydro RAZE (molecular weight of 30,000 dalton), soybean typsin inhibitor (molecular weight of 20,100 dalton), and lactalbumin (molecular weight of 14,400 dalton) were used.

(8) Isoelectric point: The isoelectric point which measured about this refined enzyme by performing isoelectric focusing (PhastSystem, PhastGel IEF 3-9 (Pharmacia manufacture)) is pI4.9. To the standard of an isoelectric point, horse myoglobin (acidic:pI6.85), Homo sapiens carbonic anhydro RAZEB (pI6.55), cow carbonic anhydro RAZEB (pI5.85), the beta-lactoglobulin A (pI5.20), a soybean trypsin inhibitor (pI4.55), and amyloglucosidase (pI3.50) were used.

[0020](9) Glucose tolerance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing glucose of 0 - 500mM concentration. The result is as being shown in drawing 3, and shows 76% of relative activity also in 500mM glucose.

(10) Ethanol resistance : in accordance with said enzyme activity measurement, relative activity was measured in 50mM phosphate buffer solution (pH 6.5) containing the ethanol of 0 - 20 capacity % concentration. It is as being shown in drawing 4, and in 10 capacity % ethanol, the result shows 85% of relative activity, and shows 67% of relative

activity in 20 capacity % ethanol.

EXAMPLE

[Example]Next, although the example of this invention is given and this invention is explained in detail, the example shown below is an example and does not limit the technical scope of this invention.

Example 1 : Culture of a strain, and 1.0% of production L-rhamnose of alpha-L-rhamnosidase, Peptone 0.5%, the culture medium which consists of 0.3% of a yeast extract and 0.3% of a malt extract was adjusted the pH to 4.0 with chloride, 100 ml of this was taught to each 40 Erlenmeyer flasks of 500-ml **, and autoclaving was carried out for 15 minutes 120 **. The Pichia ANGUSUTA (Pichia angusta)X349 share was inoculated into this, and it cultivated on the rotation agitator for two days at 28 ** and 220 rpm. The obtained culture medium 4L is centrifuged (for 1000xg and 10 minutes), biomasses are collected, and it is 20mM. A screw/tris buffers (pH 6.5) washed twice. This was suspended to the buffer solution, the 2.5-times the amount glass bead (0.45-0.55 mm in diameter) of biomass weight was added, and the biomass was crushed. Except for biomass debris, the cell extract was obtained by centrifugal separation (for 1000xg and 10 minutes). Ultracentrifuge processing (for 100,000xg and 90 minutes) of this was carried out, and it separated into the microsome fraction and the supernatant liquid fraction. Since all the activity existed in the supernatant liquid fraction when such fraction alpha-L-rhamnosidase activity was measured, the refining operation described below was presented with the supernatant liquid fraction.

[0022]Example 2: Especially refining operation shown below in refining of an enzyme was altogether performed at 4 **, unless it refused.

(1) Ammonium sulfate precipitate refined 600 ml of supernatant liquid fractions obtained in ammonium sulfate precipitate example 1. the precipitate formed from saturation in the fraction of saturation 80% 30% -- centrifugal separation (1,000xg.) It carried out for 30 minutes, collected, dissolved in the 100mM sodium acetate buffer solution (pH 6.0) containing 1M NaCl, 1mM MnCl2, 1mM CaCl2, and 0.003mM NaN3, and dialyzed to the buffer solution.

(2) The enzyme solution which carried out concanavalin A-sepharose column chromatography dialysis was given to the concanavalin A-sepharose column (phi2.6x6.5cm) beforehand equilibrated with the buffer solution. Since the target enzyme did not stick to this column, it was eluted with the buffer solution. Activity fractions are collected, and it condenses with ultrafiltration equipment (made by PM-10:Amicon), and is 20mM. It dialyzed to a screw/tris buffers (pH 6.5).

(3) DEAE Bio-Gel A They are 20mM beforehand about the enzyme solution which carried out agarose column chromatography dialysis. The DEAE Bio-Gel A agarose column (phi2.6x6.0cm) equilibrated with a screw/tris buffers (pH 6.5) was given. After the buffer solution washed, it was eluted by the sodium chloride linear gradient (0-0.3M) using the buffer solution. Activity fractions were collected and it dialyzed to 50mM sodium acetate buffer solution (pH 5.0).

[0023](4) An arabinose sepharose 6B affinity column chromatography rhamnose sepharose 6B affinity carrier, In accordance with how to recommend a manufacturing company for the epoxy activation sepharose 6B (made by Pharmacia biotech AB), rhamnose was combined and it prepared. The enzyme liquid obtained by the DEAE Bio-GelA agarose column chromatography mentioned above, The rhamnose sepharose

6B affinity column (phi1.5x7.2cm) beforehand equilibrated with 50mM sodium acetate buffer solution (pH 5.0) was given, and the buffer solution washed. Next, it was eluted with the buffer solution containing 1M NaCl, activity fractions were collected, and it dialyzed to distilled water.

(5) The enzyme solution which carried out hydroxyapatite column chromatography dialysis, The hydroxyapatite column (phi1.5x2.8cm) beforehand equilibrated with 10mM sodium phosphate buffer solution was given, the buffer solution washed enough, the back was eluted by the sodium phosphate linear gradient (10 - 200mM), and activity fractions were collected.

[0024]The activity of the enzyme in every above purification process is shown in Table 2. Cow serum albumin is used for protein as a standard substance, and it is the bottom in fixed quantity by the BURADOFODO method (Bradfordmethod).

[Table 2]

rha-sepharose 6B: Rhamnose sepharose 6B[0025]Example 3: The decomposition examination of the various chromophoric substrates shown in Table 3 was done using the refining enzymes obtained in substrate specificity example 2. The decomposition examination was done according to the method of said enzyme activity measurement, and the existence of activity was investigated by generation of p-nitrophenol. A result is shown in Table 3.

[Table 3]

[0026]Although this enzyme acts on p-nitrophenyl alpha-L-rhamnopyranoside and p-nitrophenol is separated, It hardly acts on p-nitrophenyl alpha-L-arabino furanoside, p-nitrophenyl beta-D-glucopyranoside, p-nitrophenyl beta-D-xylopyranoside, and p-nitrophenyl beta-D-cello BIOSHIDO, but it turns out that it is an enzyme with high substrate specificity.

[0027]1000 ml of grape juice (muscat OBU Alexandria kind) adjusted the pH to 7.0 by operation sodium hydroxide to the glycosides of Example 4:grape juice origin -- a synthetic adsorption resin column (phi -- 22 mm x 350 mm) Amberlite XAD-2: It let it pass to made in loam and Haas. 500 ml of distilled water washed this, and water soluble compounds were removed. After removing through and free terpenes for 1000 ml of pentane-ether (1:1) furthermore, glycosides were made eluted with 1000 ml of methanol. Concentration hardening by drying of this fraction was carried out under 30 ** and decompression, and 100 mg of glycosides were obtained. 10 mg of the above-mentioned glycosides were dissolved in 2 ml (0.5U, 50mM sodium phosphate buffer solution (pH 6.5)) of refining enzyme solutions obtained in Example 2, and it was made to react for two days at 30 **. After 5 ml of pentane-ether (1:1) having extracted the separated terpenes twice and condensing it, it analyzed on condition of the following using capillary gas chromatography. A result is shown in Table 4. Thus, the enzyme of this invention disassembles the glycosides of grape juice, and has the outstanding character to which a perfume component is made to increase notably.

[Table 4]

[0028](Analysis condition)

device: -- Hewlett Packard 5980 column: -- a DB-WAX capillary column (made by phi0.25-mmx30 m:J&WScientific)

detector: -- FID helium flow **: -- a part for 2.0-ml/-- temperature program: -- -> with a temperature up of 75 ** 220 ** (a part for 4 **/), and 220 ** (15 minutes) of maintenance sample injection section temperature: -- 230 ** primary detecting element temperature: -- 220 ** internal standard: -- amount of 2-ethyl-1-hexanol samples: -- 2microL

[0029]Example 5 :. Have a rhamnosyl group in the operation nonreducing terminal to the rhamnosyl glycoside of natural flavonoid. The decomposition product of naringin (narigin), rutin (rutin), hesperidin (hesperidin), and the rhamnosyl glycoside of quercitrin (quercitrin) was analyzed using silica gel thin layer chromatography. That is, the reaction solution in which 0.5 ml of solutions (0.5U, 50mM sodium phosphate buffer solution pH6.5) of the refining enzymes obtained in Example 2 were made to dissolve 5 mg of substrates was held at 30 ** overnight. This sample was given to 4.0microl and a silica gel thin layer (DC-Alufolien Kieselgel 60: made by Merck Co.). After developing using acetonitrile distilled water (5:1, V/V) mixture, using rhamnose and glucose as a standard substance, the ethanol solution of sulfuric acid was sprayed 10%, it heated for 5 minutes at 105 **, and sugars were detected.

[0030]As a result, naringin (narigin), the rutin (rutin), and the rhamnosyl glycoside of hesperidin (hesperidin) received decomposition by alpha-L-rhamnosidase, and rhamnose was detected. On the other hand, rhamnose was not detected about the rhamnosyl glycoside of quercitrin (quercitrin).

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1]It is a graph which shows the stable pH region and operation pH region of Pichia ANGUSUTA (Pichia angusta)X349 share of alpha-L-rhamnosidase to produce.

[Drawing 2]It is a graph which shows the stable temperature region and operative temperature region of Pichia ANGUSUTA (Pichia angusta)X349 share of alpha-L-rhamnosidase to produce.

[Drawing 3]It is a graph which shows the activity of the Pichia ANGUSUTA (Pichia angusta)X349 share alpha-L-rhamnosidase to produce, and the relation of glucose concentration.

[Drawing 4]It is a graph which shows the activity of the Pichia ANGUSUTA (Pichia angusta)X349 share alpha-L-rhamnosidase to produce, and the relation of ethanol concentration.

DRAWINGS

[Drawing 1]

[Drawing 2]

[Drawing 3]

[Drawing 4]

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(54)【発明の名称】　α－Ｌ－ラムノシダーゼおよびその製造方法

(57)【要約】　（修正有）
【課題】　高濃度のグルコースおよびエタノール存在下においても活性低下の少ない、グルコース耐性およびエタノール耐性を備えたα－Ｌ－ラムノシダーゼおよびその製造方法の提供。
【解決手段】　ピシア属に属する微生物が生産する、以下の理化学的性質を有するα－Ｌ－ラムノシダーゼ：（１）作用：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質として作用させた場合、ｐ－ニトロフェノールを遊離する。（２）ｐＨ安定性：３０℃にて３０分間保持した場合、ｐＨ５．０～７．０で安定した酵素活性を示す。（３）至適温度：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質としてｐＨ６．５で３０℃で１０分間反応させたときの至適温度は約４０℃である。（４）エタノール耐性：２０容量％エタノール中において６７％の相対活性を示す。

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【特許請求の範囲】

【請求項１】　下記の性質を有するα－Ｌ－ラムノシダーゼ。

（１）作用：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質として作用させた場合、ｐ－ニトロフェノールを遊離する。

（２）至適ｐＨ：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質として３０℃で１０分間反応させたときの至適ｐＨは６．０～７．０付近にある。

（３）ｐＨ安定性：３０℃にて３０分間保持した場合、ｐＨ５．０～７．０で安定した酵素活性を示す。

（４）至適温度：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質としてｐＨ６．５で３０℃で１０分間反応させたときの至適温度は約４０℃である。

（５）熱安定性：ｐＨ６．５にて３０分間保持したとき、２０℃まで安定した酵素活性を示し、それを超えると次第に失活する。

（６）阻害剤：ｐ－クロロ安息香酸水銀、塩化第二水銀、塩化第二銅により阻害を受け、エチレンジアミン四酢酸、メルカプトエタノールでは殆ど阻害されない。

（７）分子量：ＳＤＳ－ポリアクリルアミドゲル電気泳動法により測定した分子量は約８８，０００ダルトンである。

（８）等電点：等電点電気泳動法による等電点はｐＩ４．９である。

（９）グルコース耐性：５００ｍＭグルコース中において７６％の相対活性を示す。

（１０）エタノール耐性：２０容量％エタノール中において６７％の相対活性を示す。

【請求項２】　ピシア（Ｐｉｃｈｉａ）属に属する微生物を培養して得られる培養物から請求項１に記載のα－Ｌ－ラムノシダーゼを採取することを特徴とするα－Ｌ－ラムノシダーゼの製造方法。

【請求項３】　ピシア（Ｐｉｃｈｉａ）属に属する微生物をラムノース（ｒｈａｍｎｏｓｅ）を含む培地で培養して得られる培養物から請求項１に記載のα－Ｌ－ラムノシダーゼを採取することを特徴とするα－Ｌ－ラムノシダーゼの製造方法。

【請求項４】　請求項１に記載のα－Ｌ－ラムノシダーゼ産生能を有するピシア・アングスタ（Ｐｉｃｈｉａ ａｎｇｕｓｔａ）Ｘ３４９株（ＦＥＲＭ　Ｐ－１７２１１）。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は新規なα－Ｌ－ラムノシダーゼ、その製造方法およびその酵素を生産する微生物に関する。

【０００２】

【従来の技術】ブドウに含まれる、リナロール（ｌｉｎａｌｏｏｌ）、ゲラニオール（ｇｅｒａｎｉｏｌ）、ネロール（ｎｅｒｏｌ）、シトロネロール（ｃｉｔｒｏｎｅｌｏｌ）、α－テルピネオール（ｔｅｒｐｉｎｅｏｌ）等のモノテルペン類は、ブドウやワインの香りに大きく寄与している。これらのモノテルペン類は、芳香性の遊離体としても含まれているが、その多くは、芳香性を有しない配糖体として存在している。

【０００３】これらの配糖体の多くは、６－Ｏ－α－Ｌ－アラビノフラノシル－β－Ｄ－グルコピラノシド（６－Ｏ－α－Ｌ－ａｒａｂｉｎｏｆｕｒａｎｏｓｙｌ－β－Ｄ－ｇｌｕｃｏｐｙｒａｎｏｓｉｄｅｓ）、６－Ｏ－α－Ｌ－ラムノピラノシル－β－Ｄ－グルコピラノシド（６－Ｏ－α－Ｌ－ｒｈａｍｎｏｐｙｒａｎｏｓｙｌ－β－Ｄ－ｇｌｕｃｏｐｙｒａｎｏｓｉｄｅｓ）、６－Ｏ－β－Ｄ－アピオフラノシル－β－Ｄ－グルコピラノシド（６－Ｏ－β－Ｄ－ａｐｉｏｆｕｒａｎｏｓｙｌ－β－Ｄ－ｇｌｕｃｏｐｙｒａｎｏｓｉｄｅｓ）といったジグリコシド類であることが知られている。

【０００４】これらのジグリコシド類を酵素的に加水分解反応すると、芳香性のモノテルペン類が遊離してくる。この加水分解反応は二段階で行われる。すなわち、まず、Ｌ－ラムノシダーゼ（α－Ｌ－ｒｈａｍｎｏｓｉｄａｓｅ）、α－Ｌ－アラビノフラノシダーゼ（α－Ｌ－ａｒａｂｉｎｏｆｕｒａｎｏｓｉｄａｓｅ）、β－アピオシダーゼ（β－ａｐｉｏｓｉｄａｓｅ）などによりグリコシド結合が切断され、それぞれ対応するモノテルペニル－β－Ｄ－グルコシド（ｍｏｎｏｔｅｒｐｅｎｙｌ－β－Ｄ－ｇｌｕｃｏｓｉｄｅｓ）を生じる。次いで、このモノテルペニル－β－Ｄ－グルコシドにβ－Ｄ－グルコシダーゼ（β－Ｄ－ｇｌｕｃｏｓｉｄａｓｅ）が作用する。第一段階の反応を触媒する加水分解酵素のうち、α－Ｌ－ラムノシダーゼは、α－Ｌ－ラムノース（Ｌ－ｒｈａｍｎｏｓｅ）を含むポリサッカライドの非還元末端のα－Ｌ－ラムノピラノシド残基を加水分解する酵素である。従って、α－Ｌ－ラムノシダーゼを、β－Ｄ－グルコシダーゼなどと共に使用することにより、ワイン醸造において芳香成分を増加させて、ワインの品質を向上させることができる。

【０００５】しかしながら、ワイン醸造にこれらの酵素を利用するにあたっては、多くの克服すべき問題がある。一つには、グルコースあるいはエタノールの存在下でも酵素活性を有することが必要であることである。また、一つには、バクテリア由来の酵素の場合、好まれざるバクテリア臭があること、カビ由来の菌体外分泌型の粗酵素では、他の触媒活性のために、好まれざる副反応が起こるという問題がある。これらバクテリアやカビ由来のα－Ｌ－ラムノシダーゼについては報告があるが、一方、酵母由来のものについての報告はない。厳密に言えば、サッカロミセス・セレビジエ（Ｓａｃｃｈａｒｏｍｙｃｅｓ ｃｅｒｅｖｉｓｉａｅ）由来のものが報告されているが、その主たる触媒活性はβ－Ｄ－グルコシ

ダーゼであって、わずかにα－Ｌ－ラムノシダーゼ活性も有するというものにすぎない。

【０００６】

【発明が解決しようとする課題】従って、本発明の目的は、ワイン醸造に利用できる特性を備えた新規なα－Ｌ－ラムノシダーゼ、その製造方法およびその酵素を生産する微生物を提供することにある。

【０００７】

【課題を解決するための手段】本発明者らは、ワイン醸造に利用できる特性を備えたα－Ｌ－ラムノシダーゼを産生し得る微生物を求め、特に今まで報告例の少ない酵母を中心に探索を行なった結果、ピシア（Ｐｉｃｈｉａ）属に属する一菌株が新規なα－Ｌ－ラムノシダーゼを生産することを見出し、本発明を完成するに至った。具体的には、各種の分離源から単離した多くの微生物をＬ－ラムノースを唯一の炭素源とする培地で培養後、菌体を破砕し遠心上清画分について、後述する測定法でα－Ｌ－ラムノシダーゼ活性を測定し、目的とする微生物を選択したものである。

【０００８】すなわち、本発明によれば、下記の性質を有することを特徴とするα－Ｌ－ラムノシダーゼが提供される。

（１）作用：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質として作用させた場合、ｐ－ニトロフェノールを遊離する。

（２）至適ｐＨ：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質として３０℃で１０分間反応させたときの至適ｐＨは５．０～７．０付近にある。

（３）ｐＨ安定性：３０℃にて３０分間保持した場合、ｐＨ５．０～７．０で安定した酵素活性を示す。

（４）至適温度：ｐ－ニトロフェニル－α－Ｌ－ラムノ＊ピラノシドを基質としてｐＨ６．５で３０℃で１０分間反応させたときの至適温度は約４０℃である。

（５）熱安定性：ｐＨ６．５にて３０分間保持したとき、２０℃まで安定した酵素活性を示し、それを超えると次第に失活する。

【０００９】（６）阻害剤：ｐ－クロロ安息香酸水銀、塩化第二水銀、塩化第二銅により阻害を受け、エチレンジアミン四酢酸、メルカプトエタノールでは殆ど阻害されない。

（７）分子量：ＳＤＳ－ポリアクリルアミドゲル電気泳動法により測定した分子量は約８８，０００ダルトンである。

（８）等電点：等電点電気泳動法による等電点はｐＩ４．９である。

（９）グルコース耐性：５００ｍＭグルコース中において７６％の相対活性を示す。

（１０）エタノール耐性：２０容量％エタノール中において６７％の相対活性を示す。

【００１０】また本発明のα－Ｌ－ラムノシダーゼの製造方法は、ピシア（Ｐｈｉｃｉａ）属に属し、α－Ｌ－ラムノシダーゼを生産する微生物を培養し、得られた培養液からα－Ｌ－ラムノシダーゼを取得する方法である。

【００１１】本発明のα－Ｌ－ラムノシダーゼを生産する分離菌株の性質について以下に示す。

Ａ．形態的性質

１）乳白色のコロニーを形成する。

２）出芽により無性増殖を行う。

３）グルコース麦芽培地で１ないし４個の山高帽形の子嚢胞子を形成する。

【００１２】

Ｂ．生理的性質

１）発酵性

グルコース	＋
ガラクトース	－
マルトース	－
スクロース	－
トレハロース	＋
メリビオース	－
ラクトース	－

２）資化性

グルコース	＋	ラクトース	－
ガラクトース	－	ラフィノース	－
ソルボース	－	リビトール	＋
リボース	＋	マンニトール	＋
キシロース	＋	イノシトール	－
ラムノース	＋	メタノール	＋
スクロース	＋	エタノール	＋
マルトース	＋	硝酸塩	＋
メリビオース	－		

（＋：資化する，　－：資化しない）

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【００１３】以上の菌学的性質をもとに BARNETT & PAYNE YARROW YEASTS:Characteristicsand identification (Second edition)で検索し、この菌株をピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）に同定した。また、上述の新規なα－Ｌ－ラムノシダーゼを生産する点から、新規菌株であると判断した。従って本菌株をピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９と命名し、工業技術院生命工学工業技術研究所に寄託した。寄託番号はＦＥＲＭ　Ｐ－１７２１１である。なお、本発明に用いる微生物は、前記ピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９の他、Ｐｉｃｈｉａ属に属する微生物で本発明のα－Ｌ－ラムノシダーゼを生産する菌株であれば差し支えない。

【００１４】本発明において、α－Ｌ－ラムノシダーゼを生産する培地としては通常の微生物の培養に用いられるものでよく、使用される微生物が生育するものであれば特に制限されない。炭素源としては、例えばスクロースなどを用いることができるが、多量のα－Ｌ－ラムノシダーゼを生産する点からＬ－ラムノースが特に望ましい。窒素源としては、例えば、ペプトンがあげられる。培地には炭素源、窒素源の他、無機塩例えば塩化ナトリウムを添加することができる。培養温度は使用する微生物が生育する温度であればよいが、生育の良好な２８℃付近が好ましい。また使用する培地の初発ｐＨは４．０付近が好ましい。培養時間は当該菌の生育とα－Ｌ－ラムノシダーゼ生産に十分な時間続行されるが、通常４８時間培養することによって培養液中にα－Ｌ－ラムノシダーゼが蓄積される。

【００１５】培養後、ろ過または遠心分離またはろ過法によって菌体を集め、溶菌酵素処理あるいは超音波処理、フレンチプレス、ホモジナイザーなどの機械的破砕処理により菌体を破砕し、遠心分離して得られた上清を粗酵素液として用いる。粗酵素液はそのまま使用することもできるが、硫安塩析や溶媒沈殿法などの一般の酵素の分離精製法に準じて分離精製することができる。また、さらにイオン交換クロマトグラフィー、ゲルろ過クロマトグラフィーなどの通常の酵素の精製手段を適宜組み合わせて活性純度の高い酵素標品を調製することもできる。粗酵素液から各種クロマトグラフィー法を組み合わせて精製することにより得られた本発明酵素の性質は以下の通りである。なお、以下において、酵素活性の測定は次の方法により行った。

【００１６】［酵素活性測定法］５０ｍＭリン酸ナトリウム緩衝液（ｐＨ６．５）で希釈した酵素サンプル溶液

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０．２ｍｌに、同緩衝液に溶解した２ｍＭ　ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシド溶液０．２ｍｌを混合し、３０℃で１０分間反応させる。その後、１Ｍ炭酸ナトリウム１．６ｍｌを加えて反応を停止させ、４０５ｎｍにおける吸光度を測定することにより遊離したｐ－ニトロフェノールを定量する。また酵素活性を示す１酵素単位（Ｕ）は、上記条件、すなわち３０℃、ｐＨ６．５において、ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドから１分間に１μｍｏｌのｐ－ニトロフェノールを生成させる酵素量として定義した。

【００１７】（１）作用：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質として作用させた場合、ｐ－ニトロフェノールを遊離する。

（２）至適ｐＨ：ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドを基質とし、緩衝液として５０ｍＭクエン酸－リン酸緩衝液（ｐＨ３．０～７．０）、５０ｍＭリン酸緩衝液（ｐＨ８．０～９．０）、５０ｍＭグリシン－水酸化ナトリウム緩衝液（ｐＨ１０．０）を用いる以外は前記酵素活性測定法に従い、相対活性を測定した。その結果は図１に示すとおりであり、反応至適ｐＨは６．０～７．０付近にある。

（３）ｐＨ安定性：５０ｍＭクエン酸－リン酸緩衝液（ｐＨ３．０～７．０）、５０ｍＭリン酸緩衝液（ｐＨ８．０～９．０）、５０ｍＭグリシン－水酸化ナトリウム緩衝液（ｐＨ１０．０）の各緩衝液中で３０℃にて３０分間保持した後、前記酵素活性測定法に従い、残存活性を測定した。その結果は図１に示すとおりであり、安定ｐＨ範囲は、ｐＨ５．０～７．０である。

（４）至適温度：前記酵素活性測定法に従い、各種温度において相対活性を測定した。その結果は図２に示すとおりであり、至適温度は約４０℃である。

（５）熱安定性：５０ｍＭリン酸緩衝液（ｐＨ６．５）中で３０分間保持した後、前記酵素活性測定法に従い、残存活性を測定した。その結果、本酵素は図２に示すように、２０℃まで安定した酵素活性を示し、それを超えると次第に失活する。

【００１８】（６）各種試薬の影響：各種試薬の本酵素に対する影響を調べた。すなわち、表１に示す各種試薬を５０ｍＭリン酸緩衝液（ｐＨ６．５）に１ｍＭとなるように溶解し、これに酵素溶液を混合し、３０℃において１０分間保持した後の残存酵素活性を前記酵素活性測定法に従って測定し表１の結果を得た。

【表１】

表１．ラムノシダーゼに対する各種試薬の影響

添加化合物（１mM）	相対活性（%）
無添加	１００
$CuCl_2$	１０．９
$CaCl_2$	９８．１
$ZnCl_2$	９４．３
$FeCl_2$	１００．０
$MgCl_2$	９９．０
$CoCl_2$	９５．２
$MnCl_2$	１０１．９
$AlCl_3$	１０２．９
$HgCl_2$	６．２
EDTA	９８．１
メルカプトエタノール	９７．６
ｐ－クロロ安息香酸水銀	２．４

【００１９】（７）分子量：精製した本酵素についてＳＤＳ－ポリアクリルアミドゲル電気泳動（ＰｈａｓｔＳｙｓｔｅｍ、ＰｈａｓｔＧｅｌ　ｈｏｍｏｇｅｎｅｏｕｓ１２．５、ＰｈａｓｔＧｅｌ　ＳＤＳ　ｂｕｆｆｅｒ　ｓｔｒｉｐｓ（ファルマシア社製））を行い、測定した分子量は約８８，０００ダルトンである。分子量の標準には、ｂ型ホスホリラーゼ（分子量９４，０００ダルトン）、ウシ血清アルブミン（分子量６７，０００ダルトン）、卵白アルブミン（分子量４３，０００ダルトン）、カルボニックアンヒドロラーゼ（分子量３０，０００ダルトン）、ダイズトリプシンインヒビター（分子量２０，１００ダルトン）およびラクトアルブミン（分子量１４，４００ダルトン）を用いた。

（８）等電点：精製した本酵素について等電点電気泳動（ＰｈａｓｔＳｙｓｔｅｍ、ＰｈａｓｔＧｅｌ　ＩＥＦ　３－９（ファルマシア社製））を行い、測定した等電点は、ｐＩ４．９である。等電点の標準には、馬ミオグロビン（ａｃｉｄｉｃ：ｐＩ６．８５）、ヒトカルボニックアンヒドロラーゼＢ（ｐＩ６．５５）、牛カルボニックアンヒドロラーゼＢ（ｐＩ５．８５）、β－ラクトグロブリンＡ（ｐＩ５．２０）、大豆トリプシンインヒビター（ｐＩ４．５５）、アミログルコシダーゼ（ｐＩ３．５０）を用いた。

【００２０】（９）グルコース耐性：０～５００mM濃度のグルコースを含む５０mMリン酸緩衝液（ｐＨ６．５）中で、前記酵素活性測定法に従い、相対活性を測定した。その結果は図３に示すとおりであり、５００mMグルコースにおいても７６％の相対活性を示す。

（１０）エタノール耐性：０～２０容量%濃度のエタノールを含む５０mMリン酸緩衝液（ｐＨ６．５）中で、前記酵素活性測定法に従い、相対活性を測定した。その結果は図４に示すとおりであり、１０容量%エタノール中において８５％の相対活性を示し、２０容量%エタノール中においても６７％の相対活性を示す。

【００２１】

【実施例】次に本発明の実施例を挙げて本発明を詳しく説明するが、以下に示す実施例は一例であって本発明の技術的範囲を限定するものではない。

実施例１：菌株の培養およびα－Ｌ－ラムノシダーゼの生産

Ｌ－ラムノース１．０%、ペプトン０．５%、酵母エキス０．３%、麦芽エキス０．３%からなる培地を塩酸でｐＨ４．０に調整し、これを５００ｍｌ容の三角フラスコ４０本に１００ｍｌずつ仕込み、１２０℃、１５分間加圧滅菌した。これにピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９株を接種し、２８℃、２２０ｒｐｍにて２日間回転撹拌機上で培養した。得られた培養液４Ｌを遠心分離（１０００×ｇ、１０分間）して菌体を集め、２０mM ビス／トリス緩衝液（ｐＨ６．５）で２回洗浄した。これを同緩衝液に懸濁し、菌体重量の２．５倍量のガラスビーズ（直径０．４５～０．５５mm）を加え、菌体を破砕した。遠心分離（１０００×ｇ、１０分間）により菌体破砕物を除き、細胞抽出液を得た。これを超遠心処理（１００，０００×ｇ、９０分間）してミクロゾーム画分と上清画分に分離した。これらの画分α－Ｌ－ラムノシダーゼ活性を測定すると、すべての活性が上清画分に存在していたので上清画分を以下に述べる精製操作に供した。

【００２２】実施例２：酵素の精製

以下に示す精製操作は、特に断らない限り、全て４℃にて行った。

（１）硫酸アンモニウム沈殿

実施例１で得た上清画分６００ｍｌを硫酸アンモニウム沈殿により精製した。３０%飽和から８０%飽和の画分

にて生じた沈殿を遠心分離（１，０００×ｇ、３０分間）して集め、１Ｍ ＮａＣｌ、１ｍＭ $MnCl_2$、１ｍＭ $CaCl_2$および０．００３ｍＭ NaN_3を含む１００ｍＭ酢酸ナトリウム緩衝液（ｐＨ６．０）に溶解し、同緩衝液に対して透析した。

（２）コンカナバリンＡ－セファロースカラムクロマトグラフィー

透析した酵素溶液を予め同緩衝液で平衡化したコンカナバリンＡ－セファロースカラム（φ２．６×６．５ｃｍ）に付した。目的の酵素は、本カラムに吸着しないので、同緩衝液で溶出した。活性画分を集め、限外ろ過装置（ＰＭ－１０：Ａｍｉｃｏｎ社製）にて濃縮し、２０ｍＭ ビス／トリス緩衝液（ｐＨ６．５）に対して透析した。

（３）ＤＥＡＥ Ｂｉｏ－Ｇｅｌ Ａ アガロースカラムクロマトグラフィー

透析した酵素溶液を予め２０ｍＭ ビス／トリス緩衝液（ｐＨ６．５）で平衡化したＤＥＡＥ Ｂｉｏ－Ｇｅｌ Ａ アガロースカラム（φ２．６×６．０ｃｍ）に付した。同緩衝液で洗浄した後、同緩衝液を用いた塩化ナトリウムリニアグラジエント（０～０．３Ｍ）で溶出した。活性画分を集め、５０ｍＭ酢酸ナトリウム緩衝液（ｐＨ５．０）に対して透析した。

【００２３】（４）アラビノース－セファロース６ＢアＰフィニティーカラムクロマトグラフィー

ラムノース－セファロース６Ｂアフィニティー担体は、エポキシ活性化セファロース６Ｂ（ファルマシア・バイオテックＡＢ社製）を製造会社の推奨する方法に従い、ラムノースを結合させ、調製した。前述したＤＥＡＥ Ｂｉｏ－ＧｅｌＡ アガロースカラムクロマトグラフィーにより得られた酵素液を、予め５０ｍＭ酢酸ナトリウム緩衝液（ｐＨ５．０）で平衡化したラムノース－セファロース６Ｂアフィニティーカラム（φ１．５×７．２ｃｍ）に付し、同緩衝液で洗浄した。次に１Ｍ ＮａＣｌを含む同緩衝液で溶出し、活性画分を集め、蒸留水に対して透析した。

（５）ヒドロキシアパタイトカラムクロマトグラフィー

透析した酵素溶液を、予め１０ｍＭリン酸ナトリウム緩衝液で平衡化したヒドロキシアパタイトカラム（φ１．５×２．８ｃｍ）に付し、同緩衝液で十分洗浄し後、リン酸ナトリウムリニアグラジエント（１０～２００ｍＭ）で溶出し、活性画分を集めた。

【００２４】以上の精製工程毎における酵素の活性を表２に示す。なお、タンパク質は、牛血清アルブミンを標準物質として用い、ブラドフォード法（Ｂｒａｄｆｏｒｄｍｅｔｈｏｄ）により定量した。

【表２】

表２．ラムノシダーゼの精製

精製工程	蛋白量 (mg)	活性 (U)	比活性 (U/mg)	回収率 (%)	精製度 (倍)
粗抽出液	562.38	383.5	0.682	100.0	1.0
硫酸アンモニウム沈殿	286.46	232.3	0.811	60.6	1.2
ConA-セファロース	73.28	148.5	2.026	38.7	3.0
DEAE Bio-Gel A	8.92	121.5	13.624	31.7	20.0
rha-セファロース6B	1.65	40.8	24.727	10.6	36.3
ヒドロキシアパタイト	0.28	9.5	33.929	2.5	49.7

rha-セファロース6B：ラムノース－セファロース6B

【００２５】実施例３：基質特異性

実施例２で得られた精製酵素を用い、表３に示す各種発色基質の分解試験を行なった。なお、分解試験は前記酵素活性測定の方法に準じて行ない、ｐ－ニトロフェノールの生成により活性の有無を調べた。結果を表３に示す。

【表３】

表３．ラムノシダーゼの基質特異性

基　質	相対活性（％）
ｐ－ニトロフェニル－α－Ｌ－ラムノピラノシド	１００
ｐ－ニトロフェニル－α－Ｌ－アラビノフラノシド	６．１
ｐ－ニトロフェニル－β－Ｄ－グルコピラノシド	２．７
ｐ－ニトロフェニル－β－Ｄ－キシロピラノシド	０．２
ｐ－ニトロフェニル－β－Ｄ－セロビオシド	０．１

【００２６】本酵素はｐ－ニトロフェニル－α－Ｌ－ラムノピラノシドに作用し、ｐ－ニトロフェノールを遊離するが、ｐ－ニトロフェニル－α－Ｌ－アラビノフラノシド、ｐ－ニトロフェニル－β－Ｄ－グルコピラノシド、ｐ－ニトロフェニル－β－Ｄ－キシロピラノシドおよびｐ－ニトロフェニル－β－Ｄ－セロビオシドにはほとんど作用せず、基質特異性の高い酵素であることがわかる。

【００２７】実施例４：ブドウ果汁由来のグリコシド類に対する作用

水酸化ナトリウムでｐＨ７．０に調整したブドウ果汁（マスカット　オブ　アレキサンドリア種）１０００ｍｌを合成吸着樹脂カラム（φ２２ｍｍ×３５０ｍｍ、Ａｍｂｅｒｌｉｔｅ　ＸＡＤ－２：ローム・アンド・ハース社製）に通した。これを蒸留水５００ｍｌで洗浄し、＊水溶性化合物を除去した。さらにペンタン－エーテル（１：１）１０００ｍｌを通し、フリーのテルペン類を除去した後、メタノール１０００ｍｌにてグリコシド類を溶出させた。この画分を３０℃、減圧下にて濃縮乾固し、グリコシド類１００ｍｇを得た。実施例２で得られた精製酵素溶液２ｍｌ（０．５Ｕ、５０ｍＭリン酸ナトリウム緩衝液（ｐＨ６．５））に上記グリコシド類１０ｍｇを溶解し、３０℃にて２日間反応させた。遊離したテルペン類を５ｍｌのペンタン－エーテル（１：１）で２回抽出し、それを濃縮した後、キャピラリーガスクロマトグラフィーを用い下記の条件にて分析した。結果を表４に示す。このように本発明の酵素は、ブドウ果汁のグリコシド類を分解し、芳香成分を顕著に増加させる優れた性質を有する。

【表４】

表４．ラムノシダーゼによるグリコシド類からのテルペン類の遊離

テルペン類	ブドウ果汁由来グリコシド類１ｍｇ当り遊離量（ｎｇ） 未処理	酵素処理
リナロール	０．０	１１８０．５
α－テルピネオール	０．０	７３．５
シトロネロール	０．０	６３．５
ネロール	０．０	２１６．５
ゲラニオール	０．０	１０７５．０

【００２８】（分析条件）

装置：Ｈｅｗｌｅｔｔ　Ｐａｃｋａｒｄ　５９８０

カラム：ＤＢ－ＷＡＸキャピラリーカラム（φ０．２５ｍｍ×３０ｍ：Ｊ＆ＷＳｃｉｅｎｔｉｆｉｃ社製）

検出器：ＦＩＤ

ヘリウム流速：２．０ｍｌ／分

温度プログラム：昇温７５℃→２２０℃（４℃／分）、保持２２０℃（１５分）

試料注入部温度：２３０℃

検出部温度：２２０℃

内部標準：２－エチル－１－ヘキサノール

サンプル量：２μＬ

【００２９】実施例５：天然のフラボノイドのラムノシルグリコシドに対する作用

非還元末端にラムノシル基を有する、ナリンジン（ｎａｒｉｇｉｎ）、ルチン（ｒｕｔｉｎ）、ヘスペリジン（ｈｅｓｐｅｒｉｄｉｎ）およびクエルシトリン（ｑｕｅｒｃｉｔｒｉｎ）のラムノシルグリコシドの分解生成物をシリカゲル薄層クロマトグラフィーを用いて分析した。すなわち、実施例２で得られた精製酵素の溶液（０．５Ｕ、５０ｍＭリン酸ナトリウム緩衝液ｐＨ６．５）０．５ｍｌに基質を５ｍｇ溶解させた反応溶液を３０℃で一晩保持した。この試料を４．０μｌ、シリカゲル薄層（ＤＣ－Ａｌｕｆｏｌｉｅｎ　Ｋｉｅｓｅｌｇｅｌ　６０：メルク社製）に付した。ラムノースおよびグルコースを標準物質として用い、アセトニトリル－蒸留水（５：１，Ｖ／Ｖ）混液を用いて展開した後、１０％硫酸のエタノール溶液を噴霧し、１０５℃で５分間加熱

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して糖類を検出した。

【００３０】その結果、ナリンジン（ｎａｒｉｇｉｎ）、ルチン（ｒｕｔｉｎ）およびヘスペリジン（ｈｅｓｐｅｒｉｄｉｎ）のラムノシルグリコシドはα－Ｌ－ラムノシダーゼによって分解をうけ、ラムノースが検出された。一方、クエルシトリン（ｑｕｅｒｃｉｔｒｉｎ）のラムノシルグリコシドについてはラムノースは検出されなかった。

【００３１】

【発明の効果】本発明酵素は、高濃度のグルコースおよび高濃度のエタノール存在下においても非常に優れた活性を有しており、ワインの醸造に利用できる。すなわち、本発明酵素をβ－Ｄ－グルコシダーゼなどと共に使用することにより、ワイン醸造において芳香成分を増加させて、ワインの品質を向上させることができる。一方、バクテリア臭や夾雑酵素の働きによりワインの風味に悪影響を与えることもない。また、本酵素の、天然の＊

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＊フラボノイドのラムノシルグリコシドに対する基質特異性の広さから、ブドウ果汁のみならず柑橘類の果汁にも利用することができる。

【図面の簡単な説明】

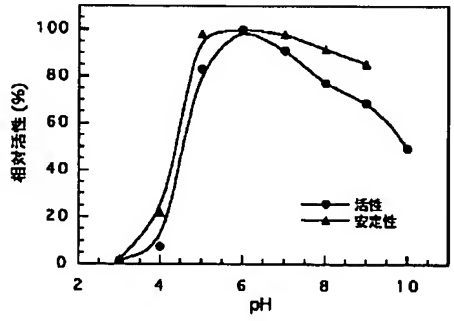
【図１】　ピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９株の生産するα－Ｌ－ラムノシダーゼの安定ｐＨ域および作用ｐＨ域を示すグラフである。

【図２】　ピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９株の生産するα－Ｌ－ラムノシダーゼの安定温度域および作用温度域を示すグラフである。

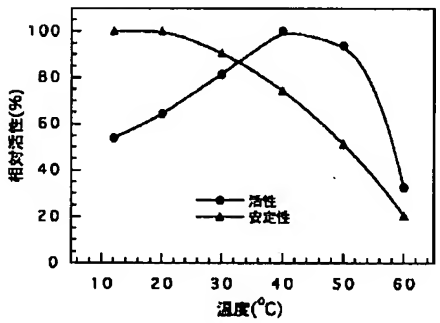
【図３】　ピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９株の生産するα－Ｌ－ラムノシダーゼの活性とグルコース濃度の関係を示すグラフである。

【図４】　ピシア・アングスタ（Ｐｉｃｈｉａ　ａｎｇｕｓｔａ）Ｘ３４９株の生産するα－Ｌ－ラムノシダーゼの活性とエタノール濃度の関係を示すグラフである。

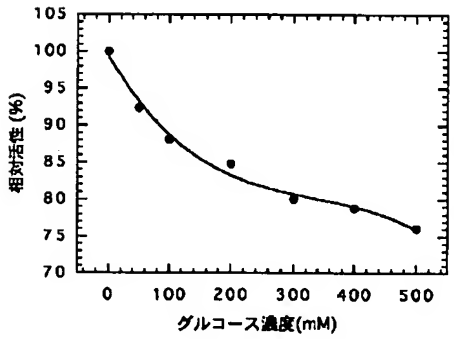
【図１】



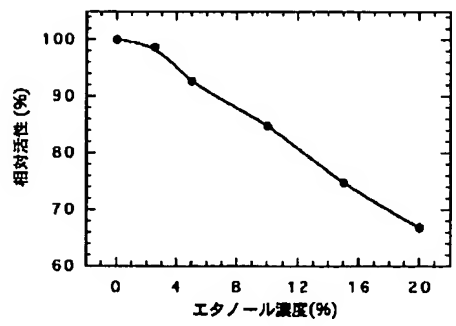
【図２】



【図３】



【図４】



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